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Novel mutations in congenital factor XII deficiency

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1. ABSTRACT

Several mutations in factor XII have been reported in patients with factor XII deficiency. Here, we described three mutations in the *F12* gene (c. 6635G>A (p. G259E), c. 6658G>C (p. R267G) and c. 8489G>A (p. E521K)) of five patients with congenital FXII deficiency. Among these, two were heterozygous mutations. All five patients had prolonged activated partial thromboplastin time, as well as markedly decreased FXII activity and antigen levels. *In vitro* studies in transiently transfected HEK 293T cells demonstrated that these mutations significantly lowered the FXII levels in the culture media, but had no impact on transcription. Further protein degradation inhibition experiments with various inhibitors suggested that the three mutants were degraded intracellularly through the proteasome pathway in the pre-Golgi compartment. Moreover, G259E and R267G mutations exhibited dominant negative effects, consistent with the phenotypes observed in the heterozygous carriers. Such dominant negative effects were not due to the dimerization of FXII. Our findings suggest that the three mutations in the *F12* gene are the causing reasons for the cross-reactive material-negative FXII deficiencies.

2. INTRODUCTION

Critically involved in blood coagulation, fibrinolysis and the complement system (1), human blood coagulation factor XII (FXII, Hageman factor) is a precursor of a multiple functional serine protease, coded by the *F12* gene that is located on the chromosomal band 5q33-qter and comprises of 14 exons with a size of 12kb (2). FXII is primarily produced by hepatocytes and secreted into circulating plasma as a mature protein with a concentration of 30~35 μg/mL and a half-life of 50~70 hours (3). The mature FXII protein is composed of 596 amino acids with an apparent molecular weight of 80 kDa, and by homologous analysis can be divided into fibronectin type I and type II homology domains, two

Table 1. Basic information of the five patients

Patient	Age	APTT (s)	PT (s)	FXII: Ag (%)	FXII: C (%)	Mutation
Patient 1	8 m	86	10.5.	NS	18	c. 8489G>GA (p.E521EK)
Patient 2	4 y	140	11.9.	4.2.	0.4.	c. 6658C>GC (p.R267RG)
Patient 3	28 y	103.6.	10.2.	3.8.	4	c. 8489G>A (p.E521K)
Patient 4	62 y	166.1.	11.5.	<1	0.6.	c. 6635G>A (p.G259E)
Patient 5	42 y	124	11.8.	<1	1.5.	c. 6658C>G (p.R267G)
APTT, activated partial thromboplastin time; PT, prothrombin time; FXII: Ag, factor XII antigen; FXII: C, factor XII activity						

growth factor domains, a kringle domain, and a catalytic domain.

FXII is originally secreted as a single chain zymogen. Upon binding to negatively charged molecules such as kaolin, dextran sulfate, sulfatide and endotoxin via its N-terminal region, FXII is cleaved between Arg353 and Val354 by plasma kallikrein, plasmin, factor XIa, FXIIa or trypsin, yielding αFXIIa, which actives FXI and initiates the intrinsic coagulation pathway. Thus, FXII has been considered to play an important role in the initiation of blood coagulation *in vitro.* Under certain circumstances such as the generation of kallikrein, αFXIIa could be further cleaved at Arg334 and Arg343, generating βFXIIa, which is enzymatically much more active than αFXIIa and could activate PK, FVII, complement system, fibrinolysis system and the kinin system via kallikrein (4).

Although FXII is considered to initiate the intrinsic coagulation pathway, it is dispensable for coagulation *in vivo* due to the lack of bleeding phenotype of the $FXII^{-/-}$ mice or FXII deficient patients. Therefore, FXII has been the only coagulation factor with unknown structure for decades, and only until recently was the crystal structure of its catalytic domain solved (5) However, despite of normal haemostasis, thrombus formation (such as arterial thrombus) in the $FXII^{-/-}$ mice is largely defective (6,7). These findings have created new interests to FXII because it raises the possibility for safe anti-thrombosis, which only targets thrombosis without influencing haemostasis (1). Therefore, FXII might be a perfect target for anti-thrombosis drug development.

Congenital FXII deficiency is a rare, autosomal recessive coagulation disease. Since FXII deficiency is asymptomatic, it is identified, in many cases, by chance due to a prolonged activated partial thromboplastin time (APTT), and the accurate incidence is not clear (8). As of December 2014, a total of 44 FXII mutations have been reported worldwide, including point mutations (missense mutations, nonsense mutations), splice site mutations, deletions and insertions. Several investigators have described the molecular basis of congenital FXII deficiency (9-15). Most mutations in the *F12* gene identified in FXII deficiency patients usually result in the lack of immunologically detectable FXII proteins, a kind of so-called cross-reactive material (CRM)-negative deficiency. In contrast, CRM-positive patients, who have considerable amount but nonfunctional FXII protein in their plasma, are more rare. In the present study, we report five Chinese patients with CRM-negative FXII deficiency. Two novel amino acid substitutions (c. 6635G>A (p. G259E) and c. 6658G>C (p. R267G)) in the kringle domain and a reported polymorphism (c. 8489G>A (p. E521K)) in the catalytic domain have been identified in the five patients, all of which lead to marked reduction of FXII levels thus the activities in the patients' plasma and cell culture media. Moreover, we observed that mutations G259E and R267G exhibited dominant negative effects, leading to FXII deficiency phenotypes in the heterozygous carriers.

3. MATERIAL AND METHODS

3.1. Patient characteristics

Five unrelated patients with FXII deficiency were included in the present study, all of whom were female. Patient 1 (8 months old), Patient 2 (4 years old), Patient 4 (62 years old) and Patient 5 (42 years old) were incidentally found to have a prolonged APTT before operation, and further coagulation screening tests showed FXII deficiency. Patient 3, a 28-year-old pregnant woman, was diagnosed with FXII deficiency during prenatal care. None of the patients had history of abnormal bleeding or thrombosis. Both FXII activities and antigen levels of the patients were determined, respectively, and the basic information of the patients was illustrated in Table 1. Other haemostatic laboratory data were normal; lupus anticoagulant (diluted Russell's viper venom time) and circulating anticoagulant against FXII (FXII inhibitor) were both negative.

3.2. Plasma FXII assays

With the informed consent, blood samples were taken from the five patients and other ten family members. Plasma FXII activity (FXII: C) was measured using one-stage clotting assay on ACL TOP (Instrumentation Laboratory, Bedford, MA, USA), and the FXII antigen level (FXII: Ag) was determined with an enzyme-linked immunosorbent assay (ELISA), in which an affinity-purified goat anti-human FXII IgG (CedarlaneLab, Canada) was used as coating antibody and a peroxidase- conjugated goat anti-human FXII IgG

(Cedarlane Lab, Canada) was used as detecting antibody. Both FXII: C and FXII: Ag levels were expressed as the percentage of the corresponding level in normal plasma pooled from 30 healthy individuals.

3.3. Polymerase chain reaction (PCR) and sequencing of the F12 gene

Genomic DNA was extracted from whole blood samples of the patients and their parents/children using a Genomic DNA extraction Kit (TianGen, China). Polymerase chain reactions (PCR) were carried out using 8 sets of specific primers that covered the entire coding region of the *F12* gene as described previously (10,15). The primer sequences, PCR mixture components and cycling conditions were available upon request. The amplified products were purified and sequenced using an ABI 3130XL sequencer (Applied Biosystems, Foster City, CA, US).

3.4. Site-directed mutagenesis and construction of the expression plasmids

FXII cDNA was ligated into pIRES2-EGFP vector after *NheI/EcoR*I cleavage to construct the FXII expression plasmid (pIRES2-FXII). Mutations (G259E, R267G and E521K) were generated in the FXII cDNA by consecutive PCR, and the resulting gene fragments were ligated into the pIRES2-FXII vector after *BssHII*/*XhoI* or *BclI*/*ScaI* cleavage. For HA-tagged and 6xHis-tagged FXII constructs, the DNA fragment encoding the HA epitope tag (YPYDVPDYA) or 6xHis epitope tag (HHHHHH) was attached, in a step-wise fashion, to the C-terminal end of FXII cDNA using regular PCR, and the resulting gene fragments were ligated into the pIRES2-FXII vector after *XhoI* and *EcoRI* cleavage. All of the gene sequences were confirmed by DNA sequencing to avoid spontaneous errors.

3.5. Cell culture and transient expression of FXII in HEK 293T cells

HEK 293T cells were maintained in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Paisley, US), supplemented with 10% fetal calf serum and 2 mM glutamine in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Cells were evenly split into six-well plates at a density of 7×10^5 cells/well a day before transfection, and transient transfection of HEK 293T cells with wild type or mutant FXII genes were carried out using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. The pIRES2 sham vector without FXII cDNA was transfected as a negative control for each transfection experiment. The cells were then grown for 48 hrs in serum-free DMEM before the conditioned media and the cells being harvested and analyzed for protein expression/activity.

3.6. SDS-PAGE and Western blot

The collected media of transfected cells were first concentrated using Amicon® Ultra-4 centrifugal filters with 10-kDa molecular weight cutoff (Millipore, USA), and then subjected to one-stage clotting assay to determine the FXII activities, or for ELISA and Western blot to determine the expression levels. For Western blot analysis, the transfected cells were collected and lysed with lysis buffer (50 mM Tris·HCl, pH 7.4., 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) containing 1x protease inhibitor cocktail for mammalian cells (Sigma). Supernatant from the cell lysates were then separated on 10% Bis-Tris SDS-PAGE, transferred to PVDF membrane, blotted with a polyclonal goat anti-human FXII IgG and a horseradish peroxidase conjugated rabbit anti-goat IgG, and detected by ImageQuant LAS 4000 mini (GE Healthcare) after incubation with chemiluminescence substrates.

3.7. Quantitative real time (qRT)-PCR for FXII mRNA level

The mRNA expression levels of wild type or mutant FXII in transiently transfected HEK 293T cells were assessed using quantitative real-time PCR (qRT-PCR). Briefly, 48 hrs after transfection, total mRNA was extracted from the cells using the QIAamp® RNA blood mini kit (Qiagen, France), and then reversely transcribed to cDNA as previously described (16). The primer sequences used for FXII amplification in qRT-PCR were 5'-CAGCTGTACCACAAATGTACCCAC-3' (forward) and 5'-AAACAGTATCCCATCGCTGG-3' (reverse) (16). qRT-PCR was performed using Qiagen Rotor-Gene Q (Qiagen, France) according to the manufacturer's protocol. An adequate fluorescent intensity was chosen and the required number of PCR cycles was defined as the threshold cycle. Results were calculated from triplicate RNA samples. The data were analyzed using $2^{-\Delta\Delta C\,(t)}$ (17).

3.8. Inhibition of protein degradation

NH_4Cl, N-acetyl-Leu-Leu-Norleucinal (ALLN), clasto-lactacystin β-lactone and Brefeldin A (Sigma Aldrich) were used as protein degradation inhibitors as previously described (18). Briefly, 24 hrs after transfection, HEK 293T cells in six-well plates were cultured in DMEM with 1% bovine serum albumin (BSA) and treated with clasto-lactacystin β-lactone (10 μM), ALLN (20 μg/mL), NH_4Cl (50 mM), Brefeldin A (10 μg/mL) or dissolving solvent (DMSO or methanol) as controls. 6 hrs after treatment, the cells were harvested and lysed in 100 μL ice-cold lysis buffer, and the FXII expression levels were determined by ELISA. Results were expressed as the percentage of the values obtained with control culture medium containing methanol or DMSO.

3.9. Co-immunoprecipitatiton of different tagged FXII

HA-tagged FXII (FXII-HA) and His-tagged FXII (FXII-His), either alone or in combination as indicated, were transiently transfected into HEK 293T cells. 48 hrs after transfection, the conditioned media were harvested and centrifuged to remove the dead cells. The media were then pretreated with 20 μL 50% Protein A-agarose

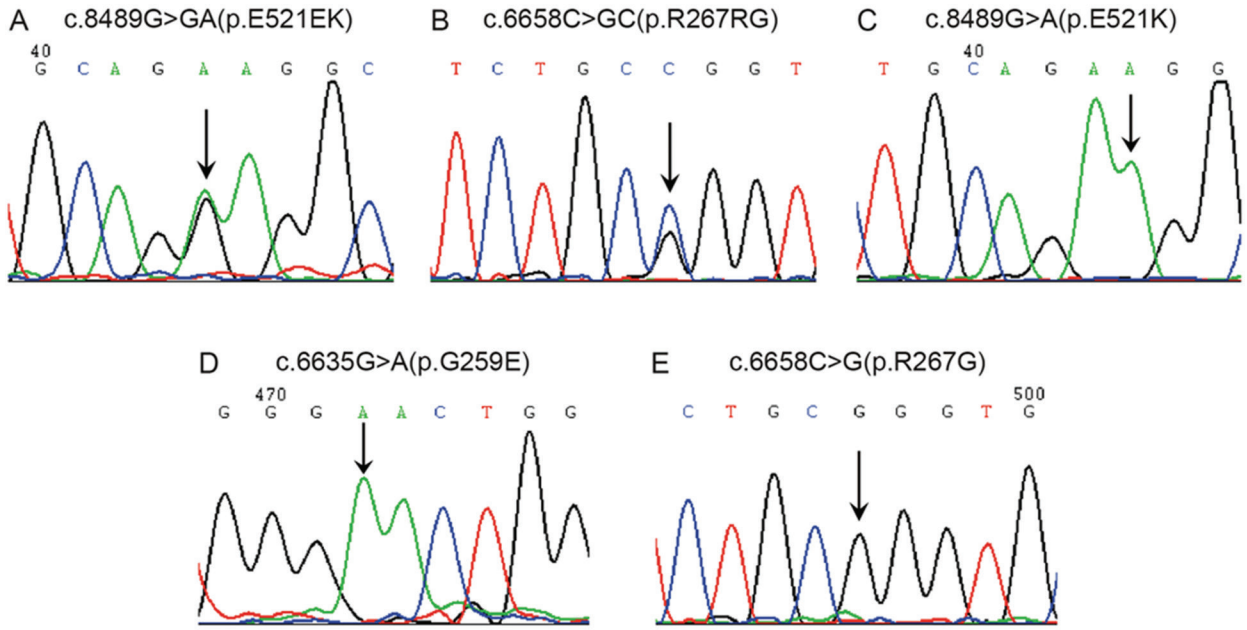

Figure 1. Sequencing results of the mutant sites of the *F12* gene in the five patients. Patient 1 (A) and 2 (B) carried a heterozygous mutation, while patient 3-5 (C-E, respectively) contain a homozygous mutation. The arrows indicated the mutated sites.

beads (Beyotime Biotechnology) at 4°C for 30min, and immunoprecipitated with 1μg indicated antibody and 20 μL 50% Protein A-agarose beads at 4°C for 30 min. The proteins bound to the beads were eluted in Tris-glycine SDS sample buffer, separated in a 10% Bis-Tris SDS gel under reducing conditions, and immunoblotted using an anti-HA antibody (Covance) or anti-His antibody (Transgen Biotech).

3.10. Statistical analysis

The data were presented as the mean ± SD calculated from multiple independent experiments. Student's t-test was used for comparison between groups, and a p value < 0.0.5 was considered to be statistically significant.

4. RESULTS

4.1. Identification of the mutations and polymorphisms in the F12 gene

Five pedigrees, all of whom were born by non-consanguineous healthy parents with an unremarkable bleeding history, exhibited FXII deficiency and were included in the present study. The clinical features and biochemical profiles of each patient were consistent with the diagnosis of congenital FXII deficiency. Except for Patient 1 showing a much lower FXII activity (18%, reference range of 50~150%), the FXII activities of the other four patients were negligible (Table 1). In addition, the FXII antigen levels (FXII: Ag) in all patients were also much lower than normal, indicating that all the patients belong to CRM-negative FXII deficiency.

Since all of the currently reported congenital FXII deficiencies were caused by mutations in the *F12* gene, we extracted the genomic DNA from the five pedigrees and sequenced the entire coding region of their *F12* gene. Nucleotide sequencing analysis showed that all five patients had missense mutations in their *F12* gene, including 3 homozygous mutations (c.6635G>A (p. G259E) in Patient 4; 6658C>G (p. R267G) in Patient 5 and c.8489G>A (p. E521K) in Patient 3), and two heterozygous mutations (c.6658C>GC (p. R267RG) in Patient 2 and c.8489G>GA (p. E521EK) in Patient 1) (Figure 1, Table 1). Sequencing results from the patient's parents or children demonstrated them as carriers of a heterozygous missense mutation (data not shown). According to the Human Gene Mutation Database, the 1000 Genomes and the Exome Variant Server database, two of the three mutations identified in the present study (*i.e.* G259E and R267G) were novel mutations while the E521K was considered as single nucleotide polymorphism (SNP). In addition, another commonly reported SNP site within the promoter region of the *F12* gene (*i.e.* 46C/T), which is considered to affect the plasma FXII antigen level, was 46 C/T in Patient 2 (data not shown). This polymorphism is common in the Japanese population and has been shown to associate with lower plasma FXII antigen level, with approximately 50% compared to the 46 C/C individuals (19).

4.2. Transient expression of wild type and mutant FXII in HEK 293T cells

To explore the molecular basis of FXII deficiency observed in the patients, plasmids containing

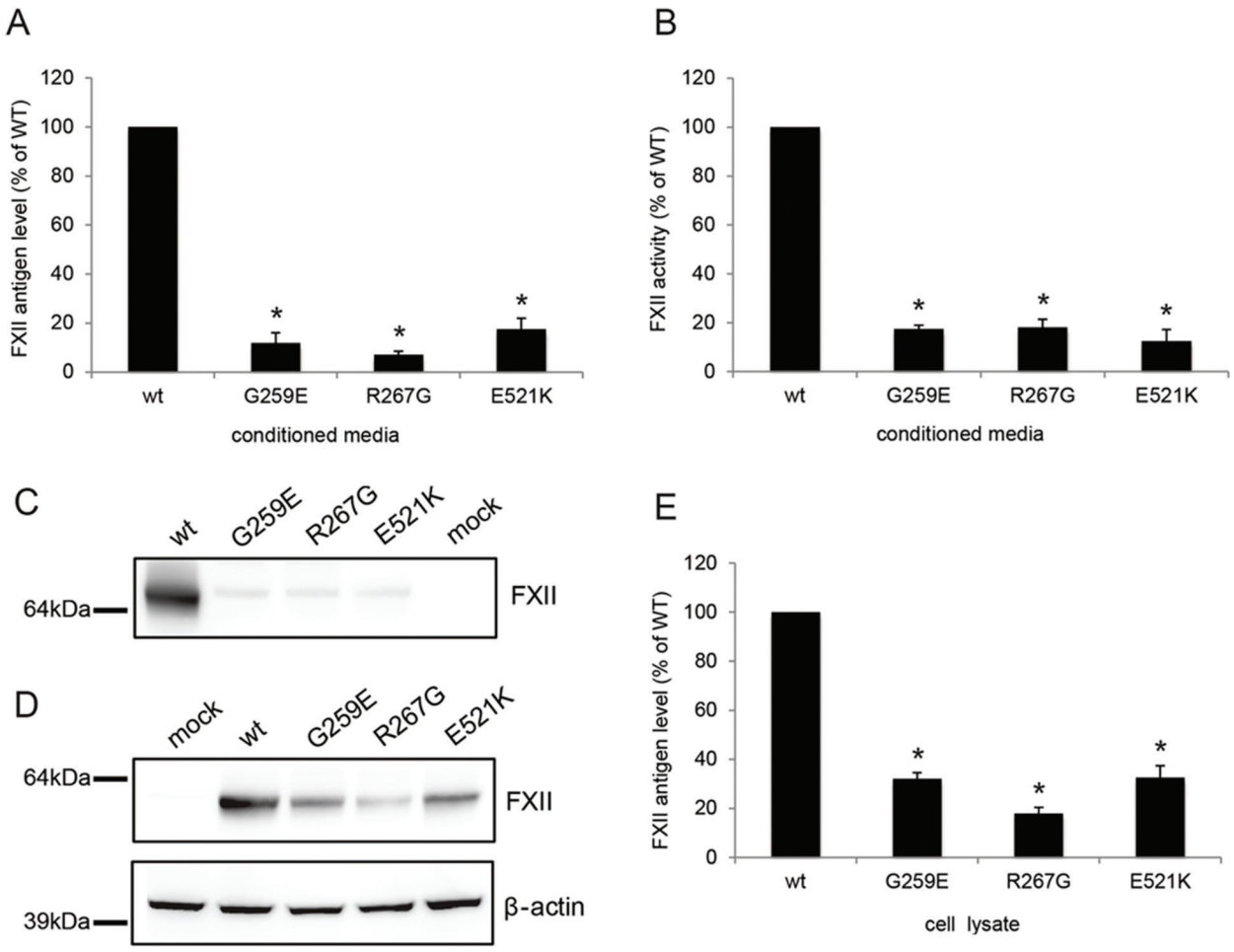

Figure 2. Expression of wild type and mutant FXII in transiently transfected HEK 293T cells. Plasmids containing wild type or mutant FXII (G259E, R267G and E521K) were transiently transfected into HEK 293T cells, and after 48 hr-culture in serum-free DMEM, the culture media and cells were collected. The FXII antigen level (A) and activity (B) in the conditioned media determined by ELISA and one-stage clotting assay, respectively. The results were calculated from 6 independent experiments and presented as the mean ± SD with the level or activity of wild type as 100%. *P < 0.0.1 compared to wild type. FXII proteins in the conditioned media (C) and cell lysates (D) detected by Western blot. The proteins were separated in a 10% Tris-Bis gel under reducing conditions, transferred to PVDF membrane, and blotted with anti-FXII or anti-actin antibody. The figure is a representative of three independent experiments. (E) The FXII antigen level in the cell lysate determined by ELISA. The results were calculated from 6 independent experiments and presented as the mean ± SD with the level of wild type as 100%. *P < 0.0.1 compared to wild type.

wild type or mutant FXII (G259E, R267G and E521K) were transiently transfected into HEK 293T cells. After 48 hr-culture in serum-free DMEM, the FXII levels and activities in the culture media of various transfected cells were determined by ELISA and one-stage clotting assay, respectively. In contrast to WT, all three mutations significantly reduced the FXII expression in the cell medium, with only 11.8. ± 4.3% (G259E), 7.0.2 ± 1.4.9% (R267G) and 17.5.3 ± 4.5% (E521K) of the wild type FXII level, respectively (Figure 2A). Similar results were also observed when FXII in the media was detected by Western blot (Figure 2C). Accordingly, the activities of the three mutants in the culture media were also significantly lowered, exhibiting only 17.4. ± 0.0.2% (G259E), 18.1.2 ± 0.0.3% (R267G) and 12.5.5 ± 0.0.5% (E521K) of WT, respectively (Figure 2B). Therefore, consistent with patients' data, our results from transfected HEK 293T cells also indicated that the low activities of the three FXII mutants were primarily due to the low expression levels of the mutant proteins in the media, rather than the mutational effects on the protein activity itself.

To explore whether the low expression levels of the three FXII mutants in the media were due to the defect in their transportation through the secretory pathway, the expression levels of wild type or mutant FXII in the cell lysates were also determined by ELISA and Western blot, respectively. Both assays showed that the cellular expression levels of the three mutants were also markedly decreased compared to wild type (32.1. ± 2.4.9% 17.9.3 ± 2.5.1% and 32.6. ± 4.7.4% of wild type for G259E, R267G and E521K, respectively,

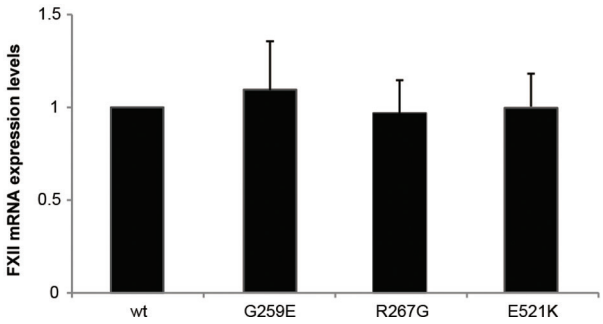

Figure 3. Comparison of mRNA expression levels between wild type and mutant FXII. Total RNA was extracted from HEK 293T cells transfected transiently with different plasmids (wild type, G259E, R267G or E521K). Quantitative RT-PCR was performed with Qiagen Rotor-Gene Q using appropriated universal probes and PCR primers. Results were calculated from three independent RNA samples and presented the mean ± SD with the level of wild type as 100%. No significant difference was observed in the mRNA expression levels between wild type and mutant FXII in HEK 293T cells.

Figure 2D and E). In addition, cellular FXII had a smaller molecular weight than that in the media (~55 kDa vs. ~80 kDa), suggesting incomplete glycosylation of the cellular FXII proteins as described previously (16). Since there was no significant amount of mutant proteins stuck inside the cells, our results suggested that the low expression levels of mutant FXII in the media were not because of the defect in transportation of the mutant proteins out of the cells.

4.3. Evaluation of FXII mRNA expression

In order to investigate whether the low expression levels of the mutant FXII were due to the defects in transcription, the mRNA expression levels of the wild type and three mutant FXII in transiently transfected cells were detected by qRT-PCR. As shown in Figure 3, no significant difference was observed in the mRNA expression values between the wild type and mutant FXII, suggesting that the three mutations had no significant impact on FXII transcription.

4.4. Effects of protein degradation inhibitors on FXII biosynthesis

Since our results showed that the three mutant FXII proteins did not significantly accumulate intracellularly, and were not affected at the transcription level, either, another potential mechanism for the reduced amounts of FXII in both the culture media and cell lysates is protein degradation. Therefore, 24 hrs after transfection, HEK 293T cells transiently expressing wild type or mutant FXII were treated with various protein degradation inhibitors for 6 hrs, and the intracellular FXII levels were then determined by ELISA. Cells maintained in medium supplemented with 1% BSA and the solvent (0.1% DMSO or 0.4% methanol) were included as controls. As shown in Table 2, NH_4Cl, a compound that inactivates lysosomal enzymes by pH modification, had no effects on intracellular levels of wild type or mutant FXII. Additionally, ALLN, a common inhibitor of neutral Ca^{2+}-dependent cysteine proteases, calpain and the 26S ubiquitin-proteasome pathway, had no effects, either. In contrast, a more specific proteasome inhibitor, clasto-lactacystin β-lactone could induce a moderate increase in the R267G level, and a much more significant increase in the wild type, G259E and E521K levels, suggesting that proteasome was involved in the intracellular degradation of the wild type and three mutant FXII proteins.

In addition, effects of brefeldin A, a compound that could block protein transportation from the ER to the Golgi complex and cause translocation of Golgi components back to the ER, on the intracellular FXII levels were also investigated (Table 2). After treatment with brefeldin A, both the wild type and mutant FXII levels significantly increased, but the increase of mutant FXII was less than wild type, suggesting that the mutants FXII proteins crossed the ER barrier less efficiently than wild type.

4.5. Dominant negative effects of the mutant FXII

Congenital FXII deficiency, like some other coagulation deficiencies, is considered as an autosomal recessive disease, and to date very a few in dominant inheritances (15). However, in the present study, we found two patients (patient 1 and patient 2) with negligible FXII levels carrying heterozygous mutations (E521EK and R267RG, respectively), whereas the other two patients (patient 3 and patient 5) carried homozygous mutations at the same point, respectively. In order to explore whether the heterozygous mutations could indeed induce FXII deficiency, 0.8. μg wild type FXII plasmids were co-transfected with 0.8. μg wild type FXII plasmids, 0.8. μg mutant FXII plasmids or 0.8. μg sham vector into HEK 293T cells, and the FXII levels in the culture media were determined by ELISA. As shown in Figure 4, addition of 0.8. μg wild type FXII plasmids could markedly increase the FXII level. In contrast, the presence of G259E and R267G both significantly reduced the FXII expression level, with 70.4. ± 11.9% and 39.4. ± 14.2% of that in cells co-transfected with wild type FXII and sham vector, respectively. On the other hand, addition of E521K to wild type FXII moderately increased FXII level, suggesting that mutations G259E and R267G, but not E521K, do exhibit a dominant negative effect. Similar results were also observed when wild type and mutant FXII were co-transfected at different ratio but keep the total amount of plasmids the same (data not shown).

4.6. FXII expressed in HEK 293T cells is not a dimer

Mutations of other coagulation factors, such as FXI, have also been reported to exhibit dominant negative effects in certain patients, and such effects were due to the formation of a heterodimer between the wild type and mutant FXI molecules (20). In order to

Table 2. Effects of the inhibitors of protein degradation and brefeldin A on the intracellular levels of FXII proteins

Treatments	Wild type	G259E	R267G	E521K
Control medium 0.4.% MeOH (n=11)	100±15.6	100±5.5	100±4.6	100±7.4
NH_4Cl (50 mM) (n=11)	124.7±26.2	96.1±6	109.1±5.1	85.4±6.1
	NS	NS	NS	NS
ALLN (20 μg/mL) (n=11)	127.3±20.6	90.7±5.2	90.3±4.5	92.7±5.9
	NS	NS	NS	NS
Brefeldin A (10 μg/mL) (n=11)	243.8±35.8	187.4±13.2	221.6±12.5	156.1±7.4
	P=0.0.0007	P=0.0.004	P=0.0.0002	P=0.0.1
Control medium 0.1.% DMSO (n=11)	100±26	100±6	100±4.9	100±5.7
Clasto-lactacystin β-lactone (10 μM) (n=11)	141.1±29.8	142.9±8	122.7±2.9	141.2±4.6
	P=0.0.2	P=0.0.001	P=0.0.4	P=0.0.2

HEK 293T cells transiently expressing wild type or mutant FXII were incubated in fresh medium containing 1% BSA, in the presence or absence of the different agents, for 6 hrs. The results were calculated from 11 independent transfections as a percentage of the values obtained from control culture medium, and presented as the mean±SD. *P* values were calculated by student's *t* test comparing the inhibitor treated samples with the control

elucidate whether the dominant negative effects induced by mutations G295E and R267G were also because of the formation of wild type/mutant FXII heterodimer, HA-tag and 6xHis-tag were appended to the C-terminus of FXII (HA-FXII and His-FXII), respectively. HA-FXII and His-FXII were transiently transfected into HEK 293T cells separately or in combination, and 48 hrs after transfection, co-immunoprecipitation with antibodies against different tags were performed to analyze whether FXII could form a dimer. As shown in Figure 5, attachment of the tags did not affect the expression levels of FXII in the media. However, anti-His antibody could only precipitate His-FXII but not HA-FXII in cells co-transfected with the two tagged FXII, and *vice versa*, indicating that FXII expressed in HEK 293T cells could not form a dimer.

5. DISCUSSION

Congenital FXII deficiency is a monogenic disease mainly caused by various mutations in the *F12* gene. Currently, around 44 genetic mutations in the *F12* gene have been reported as molecular bases for FXII deficiency, most of which are CRM negative (8,10,16,21). Since FXII deficiencies are asymptomatic, they were always identified incidentally. Similarly, the five patients in the present study were also diagnosed during regular coagulation screening before operation or during pregnancy. All probands showed typical clinical characteristics of congenital FXII deficiency, and both the FXII activities and antigen levels were greatly reduced (Table 1), demonstrating a CRM-negative trait. Additionally, since the FXII activities and antigen levels were decreased equivalently, the mutant FXII proteins in these patients might be functional variants.

Analysis of the *F12* gene discovered three mutations in the five patients—G259E, R267G and E521K, the first two of which have not yet been reported previously while the last one was considered as a polymorphism. In order to investigate the molecular etiology of the FXII deficiency observed in the patients, expression studies of the three mutant FXII were performed in transiently transfected HEK 293T cells. Compared to the wild type FXII, all three mutants exhibited significantly lowered expression levels, thus low activities in the culture media (Figure 2A-C), indicating that the three mutations, similar to many other reported CRM-negative FXII mutations (16,21), do lead to FXII deficiency. The levels of mutant FXII proteins in the culture media were slightly higher than those in the patients' plasma. Such difference might result from enforced overexpression of the mutant proteins in transfected cells. Decreased expression of the three mutants in the culture media was not due to the defects in gene transcription, as evident by comparable mRNA levels between the wild type and mutants determined by qRT-PCR (Figure 3). In addition, such decrease was not because of ER retention of the mutant proteins resulted from impaired transportation, since the cellular levels of the mutant proteins also decreased equivalently and no significant amount of proteins were trapped inside the cells (Figure 2D, E).

Several CRM-negative FXII mutations have been reported to be normally transcribed and synthesized at ER, and secretion impairment was resulted from proteasome-mediated degradation in pre-Golgi compartment (15,16,21-23). Therefore, various protein degradation inhibitors were utilized to elucidate whether the low levels of the three FXII mutants were caused by

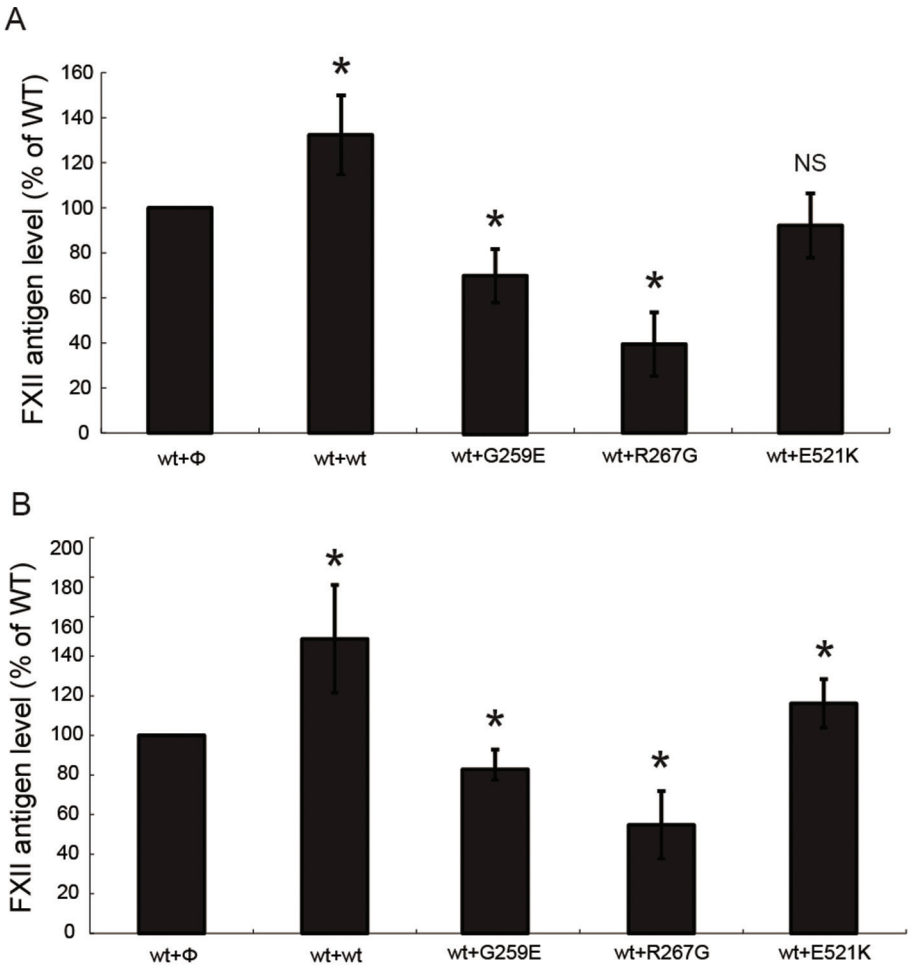

Figure 4. Detection of the dominant negative effects of the mutant FXII. The HEK 293T cells were co-transfected with 0.8. μg wild type FXII and 0.8. μg pIRES sham vector, 0.8. μg additional wild type FXII, or 0.8. μg mutant FXII. Cells transfected with 1.6. μg pIRES sham vector was included as negative control. After 48 hr-culture in serum-free DMEM, the culture media and cells were collected. The FXII antigen level in the conditioned media (A) or cell lysates (B) were determined by ELISA. The results were calculated from 6 independent experiments and presented as the mean ± SD with the level of wild type as 100%. *P < 0.0.1 compared to the FXII level in cells transfected with 0.8. μg wild type FXII and 0.8. μg pIRES sham vector.

protein degradation. Our results showed that an extensive intracellular degradation of three mutants occurred in the pre-Golgi compartment, since a more specific proteasome inhibitor (clasto-lactacystin β-lactone) but not inhibitors targeting lysosomal enzymes or cysteine proteases could significantly increase the levels of the mutant proteins (Table 2). Such protein degradation is called "ER-associated degradation (ERAD)" or "ER quality control machinery" (24,25). The three mutations (*i.e.* G259E, R267G and E521K) identified in the present study not only induced alterations in the charge, but also significant change in the side chain size. Therefore, it is reasonable to speculate that the mutations might affect the protein stability by introducing structural features into FXII, causing its recognition as mis-folded protein by ER quality control machinery, thus were degraded by proteasome after retrograde transport from ER to the cytosol through the translocon. But the molecular chaperones in ER, such as calnexin, calreticculin, and Ig binding protein (BiP), that mediate degradation of the mutant FXII proteins remained to be further investigated.

Like some other coagulation deficiencies, such as FXI deficiency, congenital FXII deficiency is also considered as an autosomal recessive disease. However, in the present study, two patients (patient 1 and patient 2) with negligible FXII levels carried heterozygous mutations (E521EK and R267RG, respectively), Co-transfection of equal amount of R267G, but not E521K, with wild type FXII significantly decreased the wild type FXII levels in the culture media, suggesting that R267G but not E521K could exhibit dominant negative effects (Figure 4). Such effect of R267G was in line with the phenotype observed in patient 2 and her father, both of whom carried the R267RG heterozygous mutation and showed marked decreased FXII activities (0.4.% and 23% of normal, respectively). On the other hand, although patient 1 who carried an E521EK mutation exhibited only

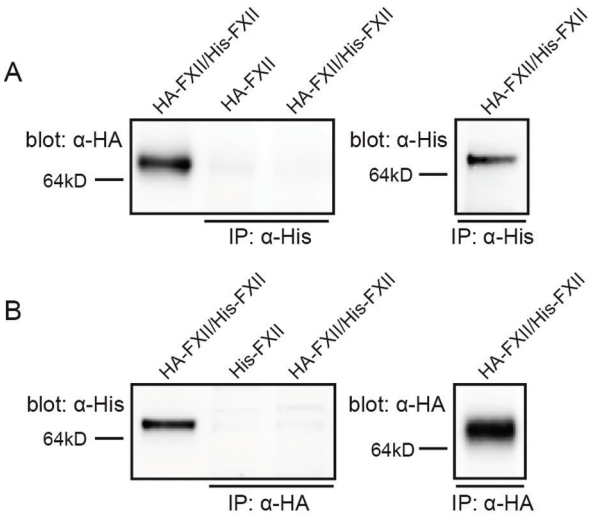

Figure 5. Co-immunoprecipitation of His-FXII and HA-FXII in transfected HEK 293T cells. Supernatant of HEK 293T cells transfected with His-FXII and/or HA-FXII were immunoprecipitated with monoclonal anti-His (A) or anti-HA (B) tag antibody and protein A agarose beads. The precipitated proteins were separated in a 10% Tris-Bis gel under reducing conditions, transferred to PVDF membrane, and blotted with anti-His or anti-HA tag antibody. The figure is a representative of three independent experiments.

18% of normal FXII activity, our *in vitro* study did not demonstrate dominant negative effects of this mutation. Further analysis on patient 3's parents, who also carried E521EK heterozygous mutation, showed that E521EK carriers had a lower, yet within normal range, FXII activity. Therefore, the significant lowered FXII activity in patient 1 might be due to her concomitant disease (the bump in right shoulder) and/or the immature development of her coagulation system at her age (8 months). In addition to R267G, we observed that G259E also exhibited a dominant negative effect, although to less extent compared to R267G (Figure 4). Consistently, the FXII activities in the children of patient 4, who carried a G259GE heterozygous mutation, were much lower than normal range (35.7.% and 36.3.% of normal, respectively). Mutations that result in the synthesis of dysfunctional polypeptides may lead to dominant negative effects by generating dysfunctional heterodimers with wild type polypeptides. Such mechanism for autosomal dominant disease has been well characterized for the multimeric coagulation proteins FXI (20), von Willebrand factor and fibrinogen (26,27). However, our results showed that, unlike FXI, FXII in the culture media of transfected HEK 293T cells could not form a dimer. Therefore, the underlying mechanisms of the autosomal dominant phenotype observed in the heterozygous mutation carriers required further investigation.

In conclusion, in the present study, we have identified three missense mutations (G259E, R267G and E521K) in five patients with FXII deficiency. *In vitro* studies suggested that these mutations might induce proteasome-mediated degradation of the mutant FXII protein in the cells, leading to the markedly lowered expression levels of FXII in the culture media, which may account for the underling mechanism for the congenital FXII deficiency in the five patients.

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7. REFERENCES

1. T. Renné, A. H. Schmaier, K. F. Nickel, M. Blombäck and C. Maas: *In vivo* roles of factor XII. *Blood* 120, 4296-4303 (2012) DOI: 10.1182/blood-2012-07-292094.
2. D. E. Cool and R. T. MacGillivray: Characterization of the human blood coagulation factor XII gene. Intron/exon gene organization and analysis of the 5'-flanking region. *J Bio Chem* 262, 13662-13673 (1987)
3. J. Bjorkqvist, K. F. Nickel, E. Stavrou and T. Renne: *In vivo* activation and functions of the protease factor XII. *Thromb Haemost* 112, 868-875 (2014) DOI: 10.1160/TH14-04-0311
4. C. Maas, J. W. P. Govers-Riemslag, B. Bouma, B. Schiks, B. P. C. Hazenberg, H. M. Lokhorst, P. Hammarström, H. ten Cate, P. G. de Groot, B. N. Bouma and M. F. B. G. Gebbink: Misfolded proteins activate Factor XII in humans, leading to kallikrein formation without initiating coagulation. *J Clin Invest* 118, 3208-3218 (2008) DOI: 10.1172/JCI35424
5. M. Pathak, P. Wilmann, J. Awford, C. Li, B. K. Hamad, P. M. Fischer, I. Dreveny, L. V. Dekker and J. Emsley: Coagulation factor XII protease domain crystal structure. *J Thromb Haemost* 13, 580-591 (2015) DOI: 10.1111/jth.12849
6. H.-U. Pauer, T. Renné, B. Hemmerlein, T. Legler, S. Fritzlar, I. Adham, W. Müller-Esterl, G. Emons, U. Sancken, W. Engel and P. Burfeind: Targeted deletion of murine coagulation factor XII gene-a model for contact phase activation *in vivo*. *Thrombo Haemost* 92, 503-508 (2004)

7. T. Renne, M. Pozgajova, S. Gruner, K. Schuh, H. U. Pauer, P. Burfeind, D. Gailani and B. Nieswandt: Defective thrombus formation in mice lacking coagulation factor XII. *J Exp Med* 202, 271-81 (2005)
DOI: 10.1084/jem.20050664

8. M. Schloesser, S. Zeerleder, G. Lutze, W. M. Halbmayer, S. Hofferbert, B. Hinney, H. Koestering, B. Lammle, G. Pindur, K. Thies, M. Kohler and W. Engel: Mutations in the human factor XII gene. *Blood* 90, 3967-3977 (1997)

9. Y. Feng, X. Ye, Y. Pang, J. Dai, X.-f. Wang and X.-h. Zhou: A novel mutation in a patient with congenital coagulation factor XII deficiency. *Chin Med J* 121, 1241-1244 (2008)

10. E. Matsuki, Y. Miyakawa and S. Okamoto: A novel factor XII mutation, FXII R84P, causing factor XII deficiency in a patient with hereditary spastic paraplegia. *Blood Co agul Fibrinolysis* 22, 227-230 (2011)
DOI: 10.1097/MBC.0b013e328343f928

11. A. M. Lombardi, E. Bortoletto, P. Scarparo, M. Scapin, L. Santarossa and A. Girolami: Genetic study in patients with factor XII deficiency: a report of three new mutations exon 13 (Q501STOP), exon 14 (P547L) and -13C>T promoter region in three compound heterozygotes. *Blood Coagul Fibrinolysis* 19, 639-643 (2008)
DOI: 10.1097/MBC.0b013e32830d8629

12. T. Miyata, S. Kawabata, S. Iwanaga, I. Takahashi, B. Alving and H. Saito: Coagulation factor XII (Hageman factor) Washington D.C.: inactive factor XIIa results from Cys-571---- Ser substitution. *Proc Natl Acad Sci U S A* 86, 8319-8322 (1989)
DOI: 10.1073/pnas.86.21.8319

13. K. Iijima, Y. Arakawa, Y. Sugahara, M. Matsushita, Y. Moriguchi, H. Shimohiro and M. Nakagawa: Factor XII Osaka: Abnormal factor XII with partially defective prekallikrein cleavage activity. *Thromb Haemost* 105, 473-478 (2011)
DOI: 10.1160/TH10-02-0123

14. W. A. Wuillemin, M. Furlan, H. Stricker and B. Lammle: Functional characterization of a variant factor XII (F XII Locarno) in a cross reacting material positive F XII deficient plasma. *Thromb Haemost* 67, 219-225 (1992)

15. T. Kanaji, S. Kanaji, K. Osaki, M. Kuroiwa, M. Sakaguchi, K. Mihara, Y. Niho and T. Okamura: Identification and characterization of two novel mutations (Q421 K and R123P) in congenital factor XII deficiency. *Thromb Haemost* 86, 1409-1415 (2001)

16. S. Oguchi, K. Ishii, T. Moriki, E. Takeshita, M. Murata, Y. Ikeda and K. Watanabe: Factor XII Shizuoka, a novel mutation (Ala392Thr) identified and characterized in a patient with congenital coagulation factor XII deficiency. *Thrombo Res* 115, 191-197 (2005)
DOI: 10.1016/j.thromres.2004.08.027

17. K. J. Livak and T. D. Schmittgen: Analysis of relative gene expression data using real-time quantitative PCR and the 2 (-Delta Delta C (T)) *Method. Methods* 25, 402-408 (2001)
DOI: 10.1006/meth.2001.1262

18. N. Enjolras, J.-L. Plantier, M.-H. Rodriguez, M. Rea, O. Attali, C. Vinciguerra and C. Negrier: Two novel mutations in EGF-like domains of human factor IX dramatically impair intracellular processing and secretion. *J Thromb Haemost* 2, 1143-1154 (2004)
DOI: 10.1111/j.1538-7836.2004.00756.x

19. G. Endler, M. Exner, C. Mannhalter, S. Meier, K. Ruzicka, S. Handler, S. Panzer, O. Wagner and P. Quehenberger: A common C-->T polymorphism at nt 46 in the promoter region of coagulation factor XII is associated with decreased factor XII activity. *Thrombo Res* 101, 255-260 (2001)
DOI: 10.1016/S0049-3848(00)00404-7

20. D. V. Kravtsov, Wenman Wu, Joost C.M.Meijers, Mao-Fu Sun, Morey A.Blinder, Thao P. Dang, Hongli Wang and David Gailani: Dominant factor XI deficiency caused by mutations in the factor XI catalytic domain. *Blood* 104, 128-134 (2004)
DOI: 10.1182/blood-2003-10-3530

21. S. Kondo, F. Tokunaga, S. Kawano, Y. Oono, S. Kumagai and T. Koide: Factor XII Tenri, a novel cross-reacting material negative factor XII deficiency, occurs through a proteasome-mediated degradation. *Blood* 93, 4300-4308 (1999)

22. H. Wada, J. Nishioka, Y. Kasai, K. Kato-Nakazawa, Y. Abe, Y. Morishita, K. Nakatani and T. Nobori: Molecular characterization of coagulation factor XII deficiency in a Japanese family. *Thromb Haemost* 90, 59-63 (2003)

23. K. Ishii, S. Oguchi, T. Moriki, Y. Yatabe, E. Takeshita, M. Murata, Y. Ikeda and K.

Watanabe: Genetic analyses and expression studies identified a novel mutation (W486C) as a molecular basis of congenital coagulation factor XII deficiency. *Blood Coagul Fibrinolysis* 15, 367-373 (2004)

24. R. R. Kopito: ER quality control: the cytoplasmic connection. *Cell* 88, 427-430 (1997) DOI: 10.1016/S0092-8674(00)81881-4

25. R. Sitia and I. Braakman: Quality control in the endoplasmic reticulum protein factory. *Nature* 426, 891-894 (2003) DOI: 10.1038/nature02262

26. S. O. Brennan, G. Maghzal, B. L. Shneider, R. Gordon, M. S. Magid and P. M. George: Novel fibrinogen gamma375 Arg>Trp mutation (fibrinogen aguadilla) causes hepatic endoplasmic reticulum storage and hypofibrinogenemia. *Hepatology* 36, 652-658 (2002)

27. S. Duga, R. Asselta, E. Santagostino, S. Zeinali, T. Simonic, M. Malcovati, P. M. Mannucci and M. L. Tenchini: Missense mutations in the human beta fibrinogen gene cause congenital afibrinogenemia by impairing fibrinogen secretion. *Blood* 95, 1336-1341 (2000)

Abbreviations: APTT, activated partial thromboplastin time; FXII: Ag, FXII antigen; FXII: C, FXII activity; MeOH, Methanol; ERAD, ER-associated degradation; ALLN, N-acetyl-Leu-Leu-Norleucinal

Key Words: Factor XII, Factor XII Deficiency, Blood Coagulation, Cross-Reactive Material-Negative, Dominant Negative

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